Adaptation of cytochrome-b_5 reductase activity and methaemoglobinaemia in areas with a high nitrate concentration in drinking-water

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An epidemiological investigation was undertaken in India to assess the prevalence of methaemoglobinaemia in areas with high nitrate concentration in drinking-water and the possible association with an adaptation of cytochrome-b_5 reductase. Five areas were selected, with average nitrate ion concentrations in drinking-water of 26, 45, 95, 222 and 459 mg/l. These areas were visited and house schedules were prepared in accordance with a statistically designed protocol. A sample of 10% of the total population was selected in each of the areas, matched for age and weight, giving a total of 178 persons in five age groups. For each subject, a detailed history was documented, a medical examination was conducted and blood samples were taken to determine methaemoglobin level and cytochrome-b_5 reductase activity. Collected data were subjected to statistical analysis to test for a possible relationship between nitrate concentration, cytochrome-b_5 reductase activity and methaemoglobinaemia. High nitrate concentrations caused methaemoglobinaemia in infants and adults. The reserve of cytochrome-b_5 reductase activity (i.e. the enzyme activity not currently being used, but which is available when needed; for example, under conditions of increased nitrate ingestion) and its adaptation with increasing water nitrate concentration to reduce methaemoglobin were more pronounced in children and adolescents.

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Introduction

Excessive nitrate concentration in drinking-water is reported to have caused methaemoglobinaemia in infants up to 6 months of age (*1–5*). Maximum permissible limits for nitrate ion in drinking-water have been set at 50 mg/l by WHO (*6*) and 45 mg/l by the Bureau of Indian Standards (IS-10500) (*7*). In several developing countries, especially India, consumption of water containing high nitrate concentrations, sometimes as high as 500 mg/l, is not uncommon.

In the body, nitrates are reduced to nitrites, causing methaemoglobinaemia. The reduction usually occurs through microbial action. The health risks from exposure to nitrates are therefore related not only to their concentration in drinking-water and food, but also to conditions conducive to their reduction to nitrites.

When nitrates are ingested, reduction to nitrites occurs in the intestinal tract, although some studies suggest that this may start in the oral cavity (*8*). Nitrite ions are absorbed into the bloodstream where they are converted back to nitrates. During this process, haemoglobin is converted to methaemoglobin. In healthy individuals, absorbed nitrates are rapidly excreted by the kidney (*9*).

The essential action in the formation of methaemoglobin is the oxidation of the ferrous ion of haemoglobin to the ferric ion, which can be brought about by the direct action of oxidants, by the action of hydrogen donors in the presence of oxygen, or by auto-oxidation (*10*). In the presence of nitrites, oxidation is direct. Normally, the methaemoglobin that is formed is reduced by the following reaction (*11*):

$$Hb^{3+} + \text{reduced cytochrome } b_5 \rightarrow Hb^{2+} + \text{oxidized cytochrome } b_5.$$

Reduced cytochrome b_5 is regenerated by the enzyme cytochrome-b_5 reductase:

$$\text{oxidized cytochrome } b_5 + \text{NADH} \xrightarrow[\text{reductase}]{\text{Cytochrome-}b_5} \text{reduced cytochrome } b_5 + \text{NAD}$$

Thus, the enzyme cytochrome-b_5 reductase plays a vital role in counteracting the effects of nitrate ingestion.

Infants constitute a vulnerable group for a number of reasons (*9*): the higher pH of the stomach (2.0–5.0) permits the growth of nitrate-reducing

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Ref. No. **0023**

organisms (*Escherichia coli, Salmonella* spp. etc.); infants have a higher consumption of water per unit body weight; fetal haemoglobin, present in infants up to 6 months of age, is more readily oxidized to methaemoglobin than adult haemoglobin; and the development of the NADH-methaemoglobin reductase system is incomplete.

While a few cases of methaemoglobinaemia in infants have been reported to be associated with water nitrate levels of less than 50 mg/l, most cases occur at levels of 90 mg/l or more (*1–5, 12*). The following clinical manifestations at different methaemoglobin concentrations have been reported (*9*): at <10%, no signs and symptoms; at 10–25%, no symptoms, cyanosis present; at 26–50%, cyanosis, dyspnoea and headache; at 51–60%, dyspnoea even on lying, cyanosis and disorientation; and at >60%, lethal consequences.

Despite the fact that, in several Indian villages, people have been consuming water containing high nitrate concentrations, at times up to 500 mg/l, no studies have been conducted so far to ascertain the prevalence of clinically manifest methaemoglobinaemia in infants. An epidemiological investigation was therefore undertaken in these areas to assess methaemoglobinaemia at different ages and to examine the protective mechanism of cytochrome-b_5 reductase activity.

Materials and methods

Water analysis was conducted in five selected village units (a part of the village served by a single source of drinking-water) in the State of Rajasthan, India, where high average nitrate concentrations were known to occur. Nitrate concentration was determined using an ultraviolet spectrophotometric screening method (*12*). The selected village units were physically close to each other, lying within a radius of 2 km. Other environmental, social and nutritional conditions in the selected village units were similar.

These village units were visited and house schedules, recording details about family members, their name, age, sex, weight, etc., were prepared. A sample of 10% of the total population from each village unit, matched for age and weight, was selected for detailed clinical and biochemical examination. A total of 178 persons were selected in five age groups: $\leqslant$1 year of age (group 1), >1–$\leqslant$8 years (group 2), >8–$\leqslant$18 years (group 3), >18 years–$\leqslant$45 years (group 4), and >45 years of age (group 5). Infants below the age of three months could not be included as a separate group for medical and ethical reasons. The number of parents willing to permit collection of blood samples from such infants was very small.

For each subject, a detailed history was recorded, a medical examination was conducted and blood samples were taken, after obtaining proper consent, for estimation of methaemoglobin (expressed as a percentage of haemoglobin) in accordance with the modified method of Evelyn & Malloy (*13*). Cytochrome-b_5 reductase activity (NADH-dependent diaphorase activity, expressed as IU per gram of haemoglobin) was determined by the methaemoglobin ferricyanide method (*14*).

Collected data were subjected to correlation and regression analysis using Microsoft Excel software to test for correlations between nitrate concentration, cytochrome-b_5 reductase activity and methaemoglobinaemia.

Results

Drinking-water nitrate concentrations of 26, 45, 95, 222 and 459 mg/l, respectively, were found in the five village units. The level of methaemoglobin and average cytochrome-b_5 reductase activity for the different age groups and nitrate concentrations are presented in Tables 1 and 2. Figures 1 and 2 show nitrate concentration versus methaemoglobin level and average cytochrome-b_5 reductase activity for the five age groups.

Methaemoglobin levels were considerably higher than 2% of the total haemoglobin concentration, the reported mean content of methaemoglobin in healthy populations (*9*). High levels of methaemoglobin were not restricted to infants only but were also prevalent in older age groups. High values were observed in all age groups at all nitrate concentrations. Maximum levels were observed at 45–95 mg/l in all age groups, with the highest value in infants (age group 1). This peak was followed by a decline in methaemoglobin and then another rising trend as nitrate concentration increased.

Clinical cyanosis was the main presentation in age group 1 but it was also present in all other age groups. Recurrent respiratory infection (40–82%) and recurrent diarrhoea (33–55%) were found in groups 1 and 2 and recurrent stomatitis (17–24%) was observed in all age groups.

Statistical analysis

Age group 1

There was good correlation between cytochrome-b_5 reductase activity and methaemoglobin levels (0.5699). Cytochrome-b_5 reductase activity showed an initial rising trend with increasing nitrate concentration and methaemoglobin levels but never exceeded 10 IU/g Hb, indicating a poor reserve of this activity in this age group. This may also account for the poor correlation between nitrate concentration and cytochrome-b_5 reductase activity (0.1179) in this age group, which has been reported by other workers also. With a further increase in nitrate concentration, the rise in cytochrome-b_5 reductase activity was followed by a decline, together with a simultaneous increase in methaemoglobin level.

Clinical cyanosis was the main presentation. The other medical problems detected were recurrent respiratory infection, recurrent diarrhoea and recurrent stomatitis.

Age groups 2 and 3

Again there was an initial rise in cytochrome-b_5 reductase activity up to 16–25 IU/g haemoglobin, which reduced methaemoglobin levels. This was followed by a decline in cytochrome-b_5 reductase activity and an increase in methaemoglobin levels.

The compensatory mechanism of cytochrome-b_5 reductase activity started at a nitrate concentration of 45 mg/l and reached a peak at about 95 mg/l. It then decreased to baseline levels by 200 mg/l. There was good negative correlation between nitrate concentration and cytochrome-b_5 reductase activity (–0.815). After an initial decrease, methaemoglobin levels rose. This rise was not affected significantly by cytochrome-b_5 reductase activity as shown by the poor correlation (0.0077 and 0.1734 in group 2 and group 3 respectively). Methaemoglobin and nitrate concentration were also poorly correlated (0.2283 and 0.0119 respectively).

Clinical cyanosis was the main presentation in both age groups. Recurrent stomatitis was also observed in both age groups and recurrent respiratory infection and recurrent diarrhoea were present in group 2.

Age groups 4 and 5

A similar pattern was observed in these age groups, although cytochrome-b_5 reductase activity never exceeded 11 IU/g Hb. There was good correlation between nitrate concentration and methaemoglobin levels (–0.5741 and –0.5816 in group 4 and group 5 respectively). The correlation between methaemoglobin and cytochrome-b_5 reductase activity was also good (0.4947 and 0.6251 respectively).

Clinical cyanosis was the main presentation in both age groups and recurrent stomatitis was also observed in both groups.

Discussion

There was good correlation between methaemoglobin level and cytochrome-b_5 reductase activity in age group 1. This correlation was poor in age groups 2 and 3, but good in groups 4 and 5. This finding indicates that in groups 1, 4 and 5, the increase in methaemoglobin levels with increased nitrate concentration is poorly compensated by cytochrome-b_5 reductase activity, whereas in groups 2 and 3 compensation is considerably better. For the lower age groups, this phenomenon can be explained by the fact that in the subjects in age group 1 (infants) development of the cytochrome-b_5 reductase system is incomplete (*9*), whereas those in age groups 2 and 3 are in the period of maximum growth and development (childhood to puberty). These observations are in accordance with the study of Keohane & Metcalf (*15*): they reported that the sensitivity of erythrocytes to oxidation of haemoglobin to methaemoglobin on exposure to nitrite gradually declined during childhood until the age of puberty, although they were unable to pinpoint the factor responsible for this phenomenon. The cause of the decline in compen-

Table 1. **Average methaemoglobin levels in five different age groups exposed to drinking-water containing varying concentrations of nitrate in Rajasthan, India[a]**

Nitrate concentration (mg/l)	Methaemoglobin (% Hb) in age group[b]				
	1	2	3	4	5
26	12.69	8.94	4.81	9.33	7.90
45	19.52	15.17	16.52	19.01	10.72
95	26.99	15.13	9.69	12.73	7.00
222	7.06	8.07	5.76	6.93	7.06
459	15.38	15.46	11.06	9.87	10.00

[a] Correlation coefficients were as follows: nitrate and methaemoglobin, 0.23; nitrate and cytochrome-b_5 reductase, -0.53 ; methaemoglobin and cytochrome-b_5 reductase, 0.70.

[b] Age group 1, ⩽ 1 year; group 2, >1–⩽ 8 years; group 3, >8–⩽ 18 years; group 4, >18 years–⩽ 45 years; and group 5, >45 years of age.

Table 2. **Average cytochrome-b_5 reductase activity in five different age groups exposed to drinking-water containing varying concentrations of nitrate in Rajasthan, India[a]**

Nitrate concentration (mg/l)	Cytochrome-b_5 reductase activity (IU per g of haemoglobin) in age group[b]				
	1	2	3	4	5
26	5.95	13.77	8.53	7.64	5.95
45	2.62	11.18	10.62	11.93	8.62
95	11.97	16.20	23.73	9.76	2.19
222	5.28	8.64	3.10	10.88	2.28
459	7.19	6.19	2.41	6.03	2.70

[a] Correlation coefficients were as follows: nitrate and methaemoglobin, 0.23; nitrate and cytochrome-b_5 reductase, -0.53; methaemoglobin and cytochrome-b_5 reductase, 0.70.

[b] Age group 1, ⩽ 1 year; group 2, >1–⩽ 8 years; group 3, >8–⩽ 18 years; group 4, >18 years–⩽ 45 years; and group 5, >45 years of age.

Fig 1. **Methaemoglobin levels in the blood versus nitrate concentration in drinking-water (all age groups)**

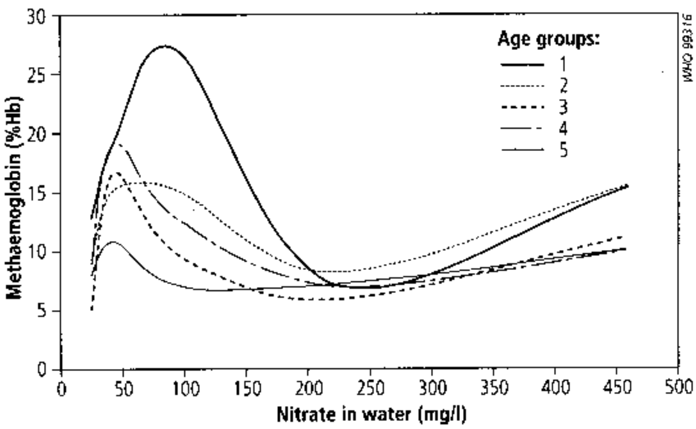
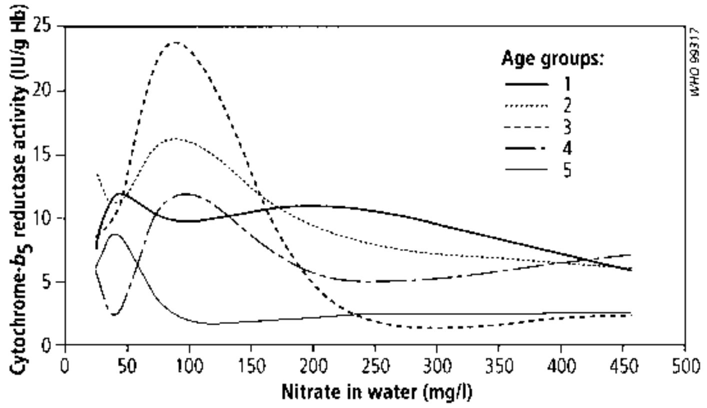


Fig 2. **Cytochrome-b_5 reductase levels in the blood versus nitrate concentration in drinking-water (all age groups)**



satory activity of cytochrome-b_5 reductase with increasing nitrate concentrations in the older age groups 4 and 5 could not be ascertained but may be the result of the saturation of the cytochrome-b_5 reductase system in these groups.

Maximum adaptation of cytochrome-b_5 reductase activity was noticed at a nitrate concentration of about 95 mg/l and decreased to the baseline level at about 200 mg/l of nitrates in drinking-water. The study indicates that high nitrate concentrations are hazardous not only to infants but also to groups over 18 years of age. The adaptation of cytochrome-b_5 reductase activity is also reduced as the nitrate concentration rises beyond about 95 mg/l making the nitrate toxicity still more pronounced at higher concentrations. The observations made in this study are in line with those of other investigators (*1–5*).

Conclusions

High nitrate concentrations in drinking-water cause severe methaemoglobinaemia in infants and adults. Children and adolescents have lower levels of methaemoglobin. A possible cause could be the reserve of cytochrome-b_5 reductase activity and its adaptation with increasing water nitrate concentration to compensate methaemoglobinaemia; the adaptation is greater in those aged 1–18 years. The adaptation reaches a maximum at a nitrate concentration of about 95 mg/l and falls back to the baseline level at about 200 mg/l. ■

Acknowledgements

This study was carried out in collaboration with the National Environmental Engineering Research Institute, Nagpur, India. The authors are grateful to Dr P. Khanna, Director, National Environmental Engineering Research Institute, Nagpur and Principal, SMS Medical College, Jaipur, and Dr S.P. Sharma, Emeritus Scientist, Council for Scientific and Industrial Research and former Director, Structural Engineering Research Centre, Ghaziabad, for their help and support. The study was partly funded by a grant from the Department of Human Resource Development Group and Extra Mural Research, Council of Scientific and Industrial Research, New Delhi, India. Help provided by the "Sookshm Vikas Sansthan", Jawanpura, Viratnagar, Rajasthan, India, in conducting the field studies is gratefully acknowledged.

Résumé

Adaptation enzymatique (activité de la cytochrome-b$_5$-réductase) et méthémoglobinémie dans les zones où l'eau de consommation présente une forte teneur en nitrates

Des cas de méthémoglobinémie ont été observés chez des nourrissons d'âge inférieur ou égal à six mois dont l'eau de boisson présentait une forte teneur en nitrates. La limite supérieure de concentration recommandée par l'OMS pour l'eau de boisson est de 50 mg d'ions nitrates par litre. Quelques cas de méthémoglobinémie ont été observés chez des enfants dont l'eau de boisson contenait moins de 50 mg d'ions nitrates par litre, mais la plupart du temps ces cas s'observent à des teneurs supérieures ou égales à 90 mg par litre. Dans plusieurs pays en développement, il n'est pas rare que l'eau de consommation soit très chargée en nitrates, avec des teneurs qui peuvent parfois dépasser 500 mg par litre. Toutefois, on constate que dans certains villages de l'Inde où les habitants consomment une eau dont la teneur en nitrates atteint quelquefois 500 mg par litre, il n'y a guère de cas de méthémoglobinémie chez les nourrissons. Nous avons donc entrepris une étude épidémiologique afin d'évaluer l'effet protecteur de la cytochrome-b$_5$-réductase vis-à-vis de la méthémoglobinémie dans les zones où l'eau de boisson présente une forte teneur en nitrates.

Nous avons choisi cinq zones de l'Etat du Radjasthan où la concentration des nitrates dans l'eau de boisson était respectivement égale à 26, 45, 95, 222 et 459 mg par litre. Nous nous sommes rendus dans chacune de ces zones et nous avons établi des fiches familiales correspondant à notre protocole statistique. Dans chacune des cinq zones, nous avons constitué un échantillon de 178 sujets (10% de la population totale) que nous avons répartis en cinq tranches d'âge en appariant les sujets sur l'âge et sur le poids. Pour chaque échantillon, nous avons établi les antécédents précis de tous les sujets qui ont ensuite subi un examen médical et une prise de sang afin de doser la méthémoglobine et de mesurer l'activité de la cytochrome-b$_5$-réductase. Les données recueillies ont été soumises à une analyse statistique destinée à mettre en évidence une relation éventuelle entre la concentration des nitrates, l'activité de la cytochrome-b$_5$-réductase et la méthémoglobinémie.

L'augmentation de l'activité de la cytochrome-b_5-réductase avec la teneur de l'eau en nitrates était plus marquée chez les 1–18 ans que chez les nourrissons et les plus de 18 ans. L'adaptation était maximale pour une teneur en nitrates d'environ 95 mg par litre et l'activité revenait à la normale aux alentours de 200 mg par litre.

Resumen

Adaptación de la actividad citocromo-b_5-reductasa y metahemoglobinemia en zonas con altas concentraciones de nitratos en el agua de bebida

Se ha informado de casos de metahemoglobinemia en lactantes de hasta seis meses como consecuencia de la presencia de altas concentraciones de nitratos en el agua de bebida. La OMS ha establecido en 50 mg/l el límite máximo permisible de nitratos en el agua potable. Aunque se han descrito algunos casos de metahemoglobinemia en lactantes a concentraciones inferiores a 50 mg de nitratos por litro de agua, la mayoría de los casos se asocian a concentraciones $\geqslant$90 mg/l. En algunos países en desarrollo el consumo de agua con concentraciones de nitratos elevadas, a veces de hasta 500 mg/l, no es un hecho infrecuente. Sin embargo, en aldeas de la India en las que la gente ha estado consumiendo agua con concentraciones de hasta 500 mg por litro se han registrado pocos casos de metahemoglobinemia en lactantes. Decidimos por tanto llevar a cabo un estudio epidemiológico para evaluar la metahemoglobinemia y el mecanismo protector de modificación de la actividad citocromo-b_5-reductasa en zonas con altas concentraciones de nitratos en el agua de bebida.

Seleccionamos en el Estado de Rajastán, en la India, cinco zonas con concentraciones promedio de nitratos de 26, 45, 95, 222 y 459 mg/l en el agua de bebida. Se visitaron esas zonas y se preparó una lista de hogares de acuerdo con el protocolo de estadísticas diseñado. Se utilizaron muestras de 178 personas (10%) de la población total en cada una de esas zonas, comparables en cuanto a edad y peso, organizándolas en cinco grupos de edad. Para cada muestra, se obtuvieron antecedentes detallados, se llevaron a cabo exámenes médicos y se obtuvieron muestras de sangre para determinar los niveles de metahemoglobina y la actividad citocromo-b_5-reductasa. Los datos reunidos se analizaron estadísticamente para determinar si existía una relación entre la concentración de nitratos, la actividad citocromo-b_5-reductasa y la metahemoglobinemia.

El aumento de la actividad citocromo-b_5-reductasa con la concentración de nitratos en el agua fue más pronunciada entre los niños y jóvenes de 1 a 18 años que entre los lactantes y la población de más de 18 años. La adaptación máxima se daba a concentraciones de unos 95 mg/l, y caía a niveles basales a concentraciones de aproximadamente 200 mg de nitratos por litro.

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